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Antisense Gene Therapy for Neurodegenerative Disease?

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Diseases resulting from defects in a single gene may be more amenable to treatment by conventional gene therapy strategies than idiopathic or polygenic disorders. We have attempted to reduce the expression *in vivo* of the Huntington's disease gene protein, Huntingtin, using an 18-mer fluoroscein-labeled phosphorothioroated antisense oligodeoxynucleotide (ODN) targeted against the start site of the first exon of the IT15 gene. Animals were given repeated intrastriatal infusions (5 μl of a 100 nmol/μl solution daily over 4 days) of the antisense ODN. The treatments ended on Day 5 and the tissue was processed for immunohistochemical and Western blot analysis. The fluorescein-labeled ODN appeared to penetrate several cells and did not cause any obvious toxicity to the neurons. The average reduction in levels of Huntingtin (16.9 ± 7.2%) did not differ significantly between striatal tissue of antisense ODN-treated animals compared to those treated with a sense ODN or vehicle. Improved methods for molecular modifications of the IT15 gene may be needed for therapeutic initiatives. © 1997 Academic Press

INTRODUCTION

Patients with progressive neurodegenerative disorders such as Parkinson's disease and Huntington's disease (HD) have limited or no effective remedial options (2, 9). Therapeutic strategies based on technology that targets such disorders at the molecular level are currently under development (16, 26). This paper discusses why certain neurodegenerative diseases may be more amenable to conventional gene therapy approaches than others, and reports results from a preliminary study using antisense oligodeoxynucleotides (ODNs) against the HD gene.

Gene therapy commonly refers to the transfer of a therapeutic gene into a target tissue and the maintenance of its function for a sufficient length of time (12). Theoretically, every disease caused by a defect in a single gene may be corrected by the transfer of the respective normal gene into the tissue(s) affected by the loss of gene function or a blockade of the faulty gene's expression. However, it is important to note that the degree and extent of dysfunction caused by defective genes may vary between tissues. Also, a further complication is that the efficacy of gene transfer may be variable between tissues. Clinical trials using gene therapy strategies have thus far been confined primarily to single-gene disorders and cancer treatment (32). As specific etiologies of neurodegenerative disorders such as Alzheimer's and Parkinson's disease have yet to be elucidated, and technologies for *in vivo* gene delivery and stability of function developed adequately, such diseases may *not* be ideal candidates for conventional gene therapy strategies. Nonetheless, correction of parkinsonism in animal models has been attempted using intracerebral transplantation of immortalized cells genetically modified to express tyrosine hydoxylase (TH), the rate-limiting enzyme in the synthesis of dopamine (19, 20). Expression of TH has also been achieved using primary cells, e.g., fibroblasts (11) and viral vectors, including the herpes simplex virus (10) and the adeno-associated virus (22). Although such studies have demonstrated successful gene transfer *in vivo,* their therapeutic relevance is limited. In all cases, expression of the transgene was transient, corresponding with the observed behavioral amelioration. Furthermore, to date, many viral-based vectors used have been found to be pathogenic (21).

Perhaps a more relevant candidate disorder for gene therapy treatment would be the autosomal dominantly inherited Huntington's disease. The mutation of the IT15 gene responsible for Huntington's disease on chromosome 4p.16.3 results in an increase in the length of a polyglutamic acid tract in the 348-kDa Huntingtin protein. The CAG repeat number in the normal IT15 gene varies, but typically ranges between 10 and 36. In contrast, in patients with HD, the number of CAG repeats range from 37 to up to 121 repeats (1). The fact that the triplet repeat expansion in IT15 does not cause a different clinical or pathological phenotype when in double dose would indicate a "gain of function" of the defective gene (36). Experiments in animals suggest that susceptibility to excitotoxic damage (particularly, glutamate receptor-mediated toxicity), oxidative stress,

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and mitochondrial dysfunction are factors involved in the specific neuronal loss observed in HD (4, 7, 28). The relationship between this pathology and the HD mutation, however, remains obscure. Moreover, the function of the normal IT15 protein has yet to be elucidated. To examine whether reduced IT15 expression affects susceptibility to glutamate receptor-mediated toxicity in neurons, we have undertaken an antisense RNA approach to modify striatal IT15 gene expression *in vivo.* It has been shown that several functions, particularly the translation of messenger RNAs, can be inhibited by antisense molecules (31, 35); thus such an approach may be useful for continuous inhibition of the expression of a target gene. Since the genetics of HD suggest a gain of function induced by the mutated IT15 gene in the pathogenesis, we attempted to modify expression of the normal IT15 gene as a basis for future work using an antisense strategy. This report describes our preliminary findings. An 18-mer antisense ODN directed against the ATG start site of the first exon of IT15 was injected directly into the murine striatum. Although fluorescein labeling of the antisense ODNs suggested that they had penetrated many striatal cells, there was only a modest reduction in striatal Huntingtin levels as assessed by immunostaining and Western blots.

METHODS

A synthetic 18-mer antisense ODN complementary to bases 75–92 of the murine IT15 sequence and designed to block translation of mouse IT15 mRNA was synthesized (OPERON Technologies, Inc., Alameda, CA). The ODN was phosphorothioroated at all positions to prevent degradation by nucleases and florescein labeled at the 5′ end so that uptake and incorporation by the cells could be easily visualized. The sequence of the antisense ODN was selected to have a base composition providing an appropriate affinity with IT15 mRNA at body temperature and salt concentration. Also, sequences with significant potential to form secondary structures were excluded and no homologous sense sequences were found in the GenBank database (GCG Sequence Analysis Software Package—Genetics Computer Group, Inc.). The antisense ODN used for this study was 5′ CAT GAC GGC TTC CTG CCC 3′, and a "sense" ODN with the base composition 5′ GGG CAG GAA GCC GTC ATG 3′ served as a control (Fig. 1).

Fifteen animals (CD-1 mice—Charles River, MA) were divided into three groups ($n = 5$): Antisense-treated, sense-treated, and control (vehicle was distilled water). Five microliters of 1 mM solutions of

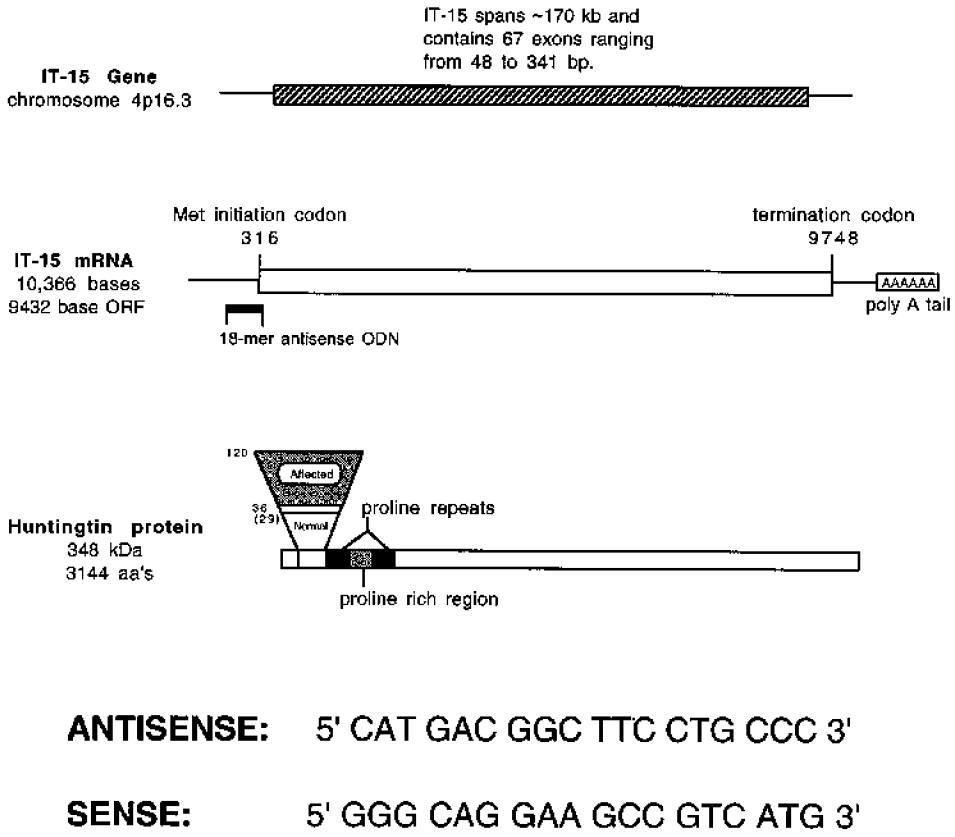

FIG. 1. Chromosomal location and structure of IT15. An 18-base oligonucleotide complementary to the region 5′ of the translation site is indicated. The CAG repeat region is found in the first exon and the ODN sequences used for the experiment are shown.

antisense or sense ODNs or 5 μl of water was directly injected into the striatum of adult CD-1 mice (coordinates: AP +0.7, L −1.5, V −2.6) (25). Animals received daily infusions of ODNs or vehicle administered stereotaxically for 4 consecutive days. The treatment volume was infused over 20 min and there was a further wait of 10 min before the needle was retracted from the striatum. On the fifth day, mice were sacrificed and tissue was processed for immunostaining or Western blotting.

Immunohistochemistry. Animals were transcardially perfused with 50 ml of cold heparinized saline (1000 units heparin/liter 0.9% saline) followed by 50 ml of cold 4% paraformaldehyde dissolved in 100 m*M* phosphate-buffered saline (PBS; pH 7.4) under deep barbiturate anesthesia. Brains were removed and post-fixed for 8 h in 4% paraformaldehyde before being transferred to a solution of 30% sucrose in PBS. Following dehydration, brains were sectioned on a freezing sledge microtome at 40 μm. Sections were mounted onto slides (Fisher Scientific) and stained with cresyl violet to visualize cell bodies or were processed as free-floating sections for IT15 immunohistochemistry.

A polyclonal anti-Huntingtin antibody directed against peptide positions 3114–3141 was the primary antibody used for immunostaining (18). Sections were pretreated with 50% methanol and 0.3% hydrogen peroxide in PBS for 20 min, rinsed three times in PBS, and then preincubated for 1 h in 10% normal blocking serum (NBS; normal goat serum in PBS). Sections were

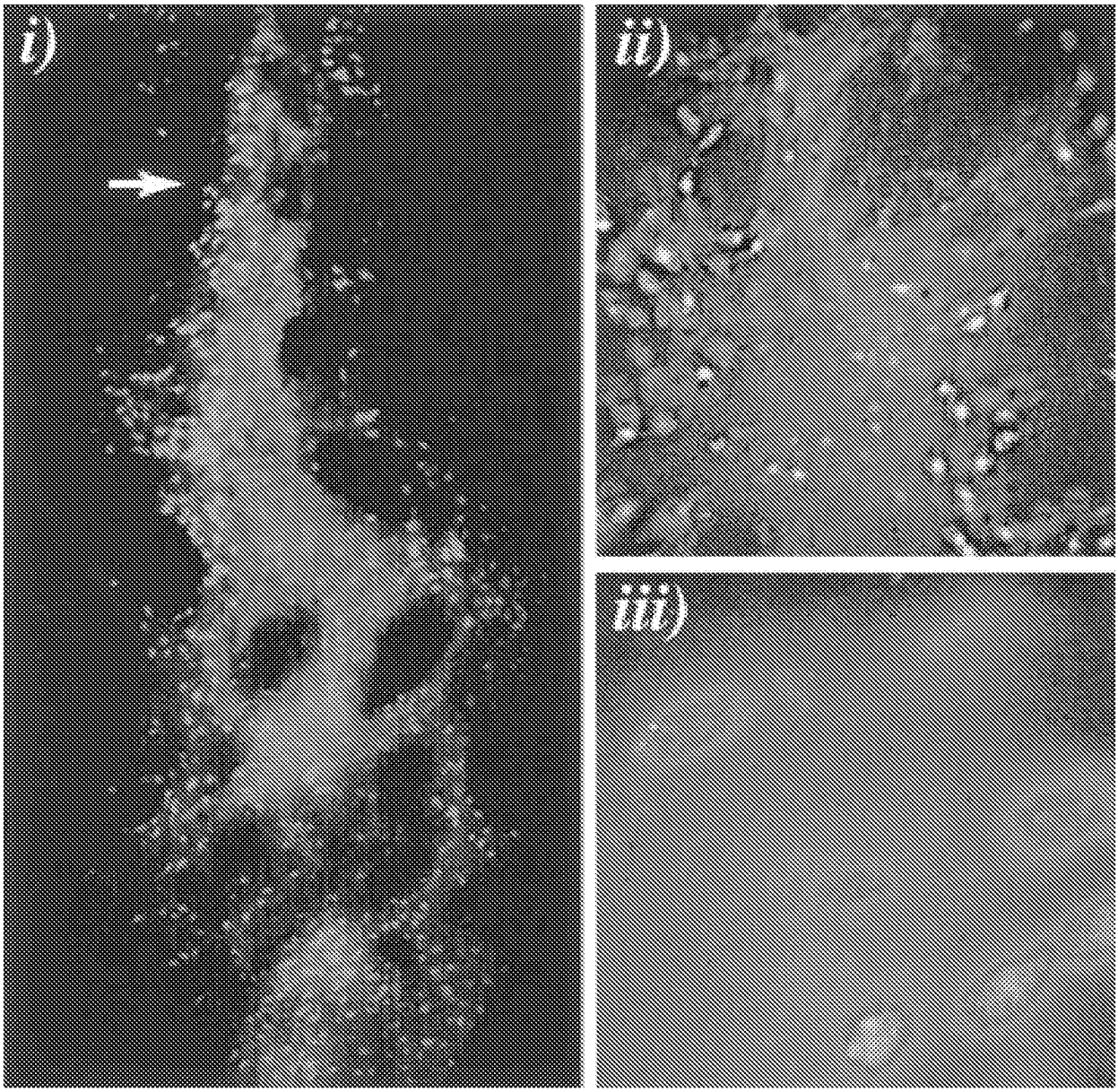

FIG. 2. *In vivo* use of antisense ODNs to reduce IT15 expression. (i) Uptake of 10 nmol of fluorescein-labeled antisense IT15 ODN by striatal cells at 24 h postinjection. [Magnifications: (*i*) 10×, (*ii*) 25×, (*iii*) 40×.]

incubated overnight with the primary antibody at a 1:1200 dilution in PBS containing 1% normal goat serum, 1% bovine serum albumin, and 0.1% Triton-X 100. Sections were then washed with PBS, incubated with goat anti-rabbit biotinylated secondary antibody (Vector Labs) diluted 1:200 in 2% NBS for 90 min, and rinsed once in PBS and twice in 0.05 *M* Tris-buffered saline (TBS). They were then developed with 0.04% hydrogen peroxide and 0.05% 3,3′-diaminobenzidine (Sigma) in TBS for 0–5 min. Sections were mounted onto subbed slides, allowed to air dry, dehydrated in graded alcohols, cleared in xylene, and finally coverslipped using Permount (Fisher Scientific).

Western blotting. Fresh-frozen samples of mouse striata were taken using a brain punch (2 mm^3), the cells were lysed, and the amount of protein extracted was measured using the BSA protein assay (Bio-Rad). Equal amounts of protein (20 μg) per lane were loaded onto a 6% or gradient polyacrylamide gel, and electrophoresis was performed according to standard protocols. The nitrocellulose membranes were initially blocked with 5% milk in Tris-buffered saline with 0.1% Tween 20 (TBS-T); they were then washed twice in TBS-T and incubated at 4°C overnight in a 1:5000 dilution of the primary anti-IT15 antibody in PBS (Chemicon MAB 2166, directed against peptides 181–812 of Huntingtin). Next, sections were washed five times in TBS-T and incubated at room temperature for 60 min in a 1:5000 dilution of a horseradish peroxidase-labeled antimouse antibody (Amersham) in PBS. The membranes were washed again in TBS-T and the signal was visualized using the ECL kit as described by the manufacturer (Amersham). Density of the bands was quantified using the computer software NIH Image (Version 1.59). The data were analyzed using a one-way analysis of variance (Statworks—Macintosh).

RESULTS

To confirm that the ODNs were incorporated into striatal cell bodies, the synthetic polymers were fluorescein labeled at their 5′ terminus. Uptake of the fluorescein-labeled antisense IT15 ODN by striatal cells 24 h after the last infusion was evident (Fig. 2). Fluorescent cells were seen up to 100 μm from the needle tract. To evaluate the morphological effects of repeated intrastriatal infusions, Nissl staining was performed. The site of injection was in the center of the striatum and there was no evidence of neuronal loss or gross damage to the striatal parenchyma in all three experimental groups (Fig. 3).

Immunohistochemical staining with an anti-Huntingtin antibody (18) (Fig. 4) demonstrated a cytoplasmic and nuclear localization of Huntingtin. Histological analysis at high magnification, however, revealed no detectable reduction in Huntingtin levels around the injection site of animals treated with IT15 antisense

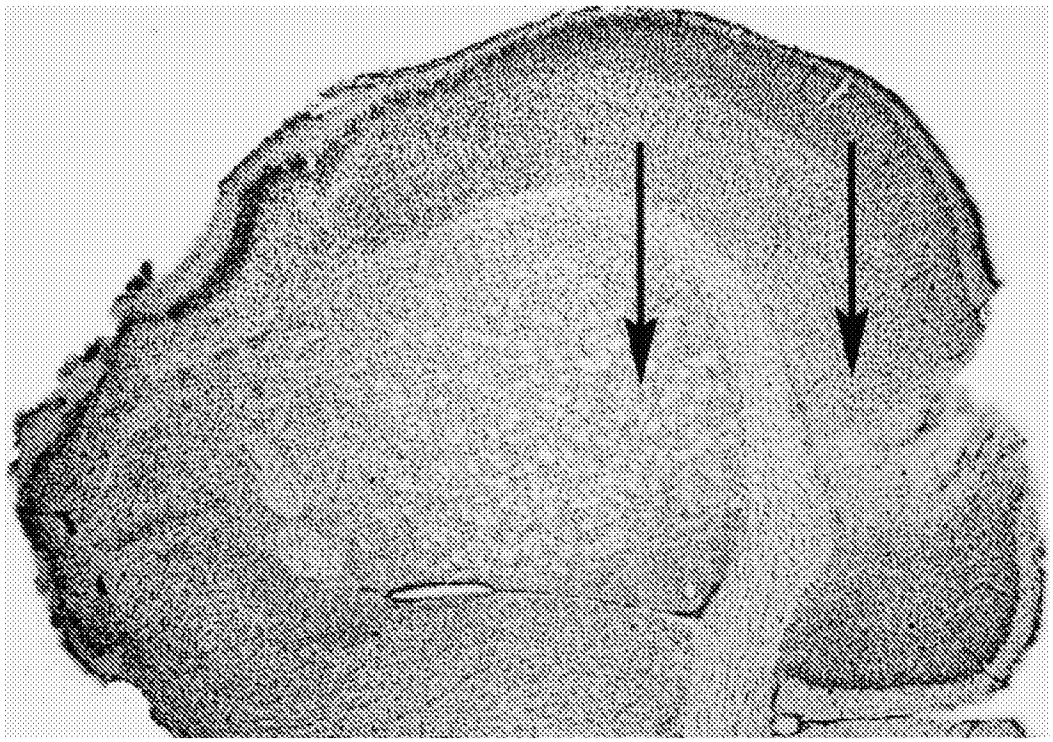

FIG. 3. Nissl stain 5 days after repeated intrastriatal infusions of antisense IT15 ODNs. (Magnification 5×.) *Note:* No evidence of neuronal loss or gross damage to the striatum.

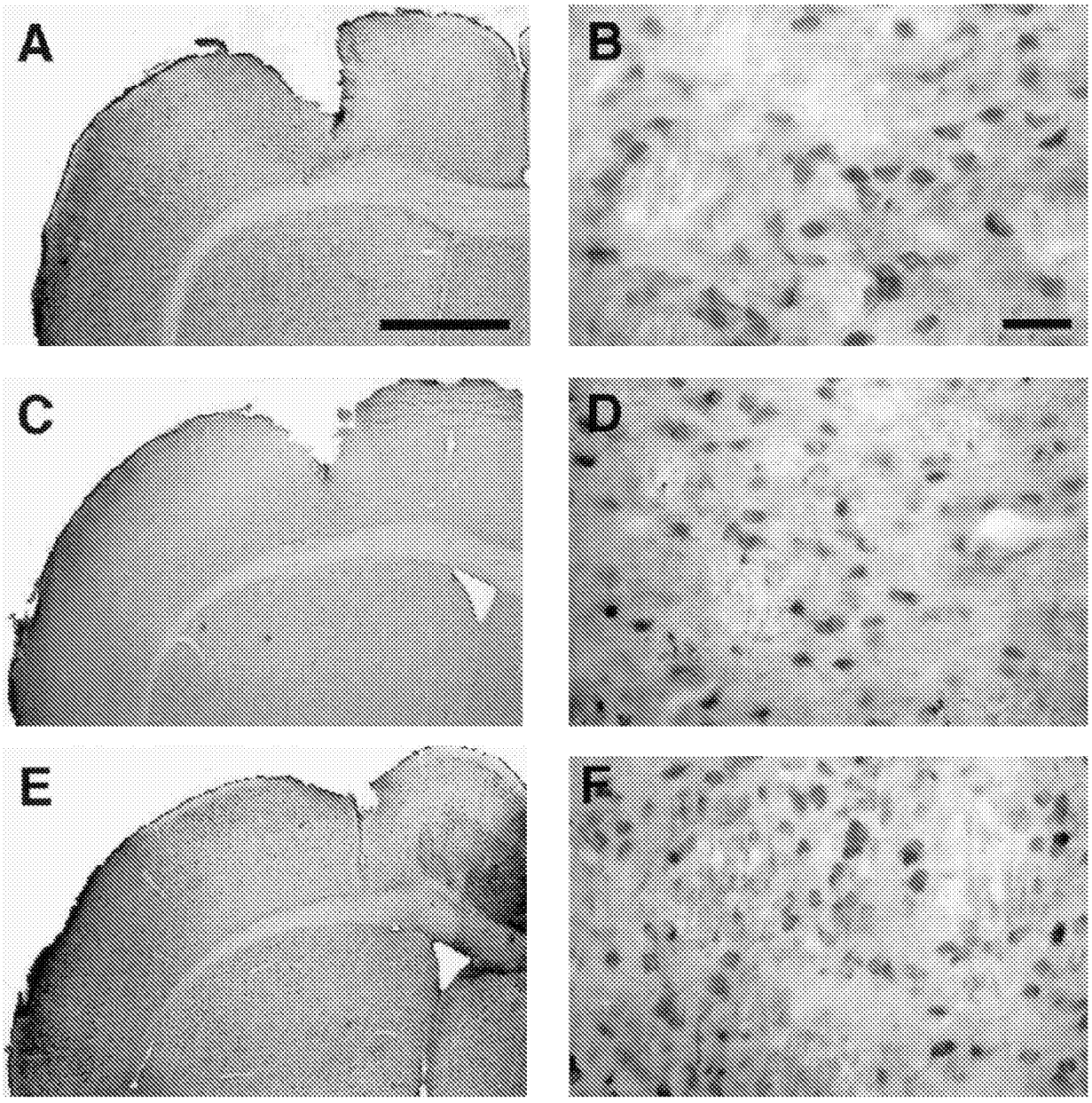

FIG. 4. IT15 immunostaining of the infused striatum showing no difference in intensity of staining between the experimental groups. Antisense-treated animal (A and B), sense-treated animal (C and D), and vehicle-treated animal (E and F). (A, C, and E) 5×; scale bar, 1 mm. (B, D, and F) 40×; scale bar, 0.5 mm.

(Figs. 4A and 4B) or sense (Figs. 4C and 4D) ODNs compared to vehicle-treated controls (Figs. 4E and 4F).

To further assess and quantify any reduction effects of antisense treatment on striatal protein levels, Western blotting was performed. For this purpose a "striatal tissue punch" (2 mm^3) was taken from the fresh brain of the animal and processed according to standard methods for analysis. Animals treated with IT15 antisense ODNs (Fig. 5, lanes A-1–A-3) had an average reduction of 16.9 ± 7.2% (statistically nonsignificant) in levels of Huntingtin compared to animals treated with the sense sequence (Fig. 5, lanes S-6 and S-8) and controls (Fig. 5, lanes C-1 and C-2). All Western blots were run in duplicate to ensure reliability of the observed effect.

DISCUSSION

Due to the fact that a gain of function hypothesis is strongly implicated in the pathogenesis of HD and that it is a single-gene disorder, it was thought that an antisense approach to block or reduce expression of the aberrant IT15 gene would be useful in uncovering its role in neuronal death. As a first step, we used antisense ODNs in an attempt to reduce normal IT15

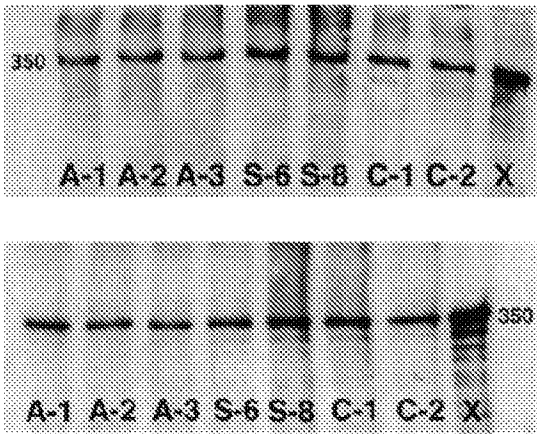

FIG. 5. Western blots showing IT15 protein levels in the striata of experimental animals. There was no significant difference between the experimental groups. Animals treated with the IT15 antisense ODN showed a 16.9 ± 7.2% reduction in Huntingtin levels compared to animals treated with the sense sequence. Antisense ODN, A-1, A-2, A-3; sense ODN, S-6, S-8; distilled water, C-1, C-2; and X, whole mouse brain.

expression. Repeated injections of a phosphorothiorated 18-mer ODN targeted against the initiation codon of the IT15 gene into the striatum of mice did not result in a significant decrease in levels of Huntingtin as assessed by immunostaining and/or Western blot analysis. Other investigators have also had difficulty in attenuating IT15 expression using antisense strategies (Dr. S. Hersch—personal communication), suggesting that the IT15 gene may not be as amenable to this approach as other genes, for instance, *c-fos* (8). There may be several explanations for such an observation. The IT15 protein is a large stable protein with a low turnover rate; its half-life is believed to be greater than 48 h. To account for the long half-life of Huntingtin, we gave repeated daily injections of the ODNs so that an accumulation of effect might be observed. Also, a relatively large final volume and concentration of ODN was infused into the striatum: 20 μl of a 100 nmol/μl solution over 4 days; however, it appears that these measures were not sufficient to achieve the desired effect. Alternatively, although the sequence of the antisense ODN overlapped the initiation codon for the IT15 gene and was designed to incorporate standard parameters for such molecules, it may have been inappropriate. Finally, the possibility that the ODNs used, although chemically modified for resistance, were degraded by endogenous nucleases and the fluorescein signal detected in the cells was simply a fragment of the 18-mer cannot be discounted.

Although traditional antisense research has focused on cancer and viral infections, recent advances in antisense technology have made the application of this methodology attractive for its therapeutic potential in the treatment of neurodegenerative disorders (38). Initially, one of the greatest advantages of the application of antisense ODNs to biological systems was the inherent selectivity conferred by sequence-specific nucleic acid hybridization, thus specifically altering the biosynthesis of only one target protein. Antisense ODNs have the potential to disrupt protein synthesis at several different levels. These include RNA splicing, blocking, transcription, distortion of the secondary and tertiary structures in the mRNA which form recognition and binding sites for nonribosomal proteins involved in RNA processing, transport, regulation of translation, and stabilization (5, 33). Much effort has been applied to increasing the effectiveness of antisense agents, e.g., by the chemical modification of oligonucleotides to make them increasingly resilient to nucleases which are present in serum (34) and the coupling of oligomers to polycations such as polylysine to resolve the problems of cellular uptake and trafficking (13). Several studies have reported selective effects of antisense agents in culture, including the inhibition of HIV-1 replication by a high-copy-number vector expressing antisense RNA for reverse transcriptase (23), antisense oligonucleotide inhibition of acetylcholinesterase gene expression (29), and elimination of potassium channel expression in a pituitary cell line (6). Although antisense effects in culture do not necessarily extrapolate directly to the *in vivo* situation, there have been a number of demonstrations of *in vivo* efficacy of antisense ODNs (8, 13, 24). There is virtually no uptake of oligonucleotides from the circulation into the CNS (5). However, under experimental conditions, ODNs may be injected stereotactically into the brain near the structures under functional investigation (8, 17), and for therapeutic applications it has been shown that adequate uptake into the parenchyma may be achieved by continuous infusion into the CSF or ventricles (35, 37).

Despite the remarkable effects observed in a variety of paradigms reported in the literature and a rapid transit to clinical trials (3), the results from application of antisense agents to various biological systems have not been without contention (15, 30). It has been found that non-sequence-specific interactions between ODNs and proteins and/or nucleic acid targets may result in a number of biological effects (31). One of the first studies to claim an *in vivo* effect using an antisense phosphorothiorated analogue targeted *c-myb* and inhibited intimal arterial smooth muscle cell accumulation in rat carotid artery (27). It has subsequently been shown that this result was predominantly non-sequence specific (6). Similar reports of nonspecific interactions have highlighted the importance of the stringent controls in antisense experiments and the need for elucidation of the mechanisms operating in such systems (30, 34).

Debate over the existence and reliability of antisense

effects for the modification of gene expression notwithstanding, we initiated experiments testing methods for antisense oligonucleotide delivery into the neostriatum. In conclusion, fluorescein labeling suggested that the ODNs had penetrated many striatal cells and Nissl staining indicated that the phosphorothiorated ODNs used were not toxic to the brain. However, immunohistochemical analysis or Western blots of striatal tissue samples revealed no significant difference in the levels of Huntingtin between animals treated with IT15 antisense or sense ODNs compared to control animals. Thus the 18-mer antisense ODN complementary to bases 75–92 of the murine IT15 sequence in our hands had no effect on striatal levels of the IT15 protein. However, continual advances in oligonucleotide therapeutics (24, 14) suggest that, with refinement, this approach may result in functional effects also when applied to neurodegenerative diseases.

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